NETWORK PHARMACOLOGY COMBINED WITH CHROMATOGRAM SIMILARITY ANALYSIS FOR BIOMARKERS EXPLORATION OF DULIANG HERBAL FORMULATION

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Abstract

Objectives: To explore potential biomarkers of the Duliang herbal formula (DHF). ***Methods:*** The DHF consisting of a radix of *A.dahurica* and a rhizome of *L. chuanxiong* was investigated the pharmacological mechanism for migraine treatment via a network pharmacology approach using published databases and software. Besides, an experimentally chromatographic analysis of DHF was performed to identify potential active components. A combination of two approach results could contribute to propose biomarkers for quality control of DHF. ***Results:*** To extend our previous *in silico* study on anti-migraine compounds in DHF via network pharmacology approach, chromatogram similarity analysis of DHF with different origin herbs had carried out and showed that these active compounds, namely ferulic acid, senkyunolide A, senkyunolide I, ligustilide, byakangelicol, oxypeucedanin, imperatorin, isoimperatorin, and oxypeucedanin hydrate, could be considered as quality markers of formula. ***Conclusion:*** Potential biomarkers of DHF for anti-migraine can be explored via an integrated approach using network pharmacology and chromatographic analysis.

Keywords: Network pharmacology; Chromatography; Biomarker; Duliang; Migraine.

INTRODUCTION

A migraine is defined as a disabling neurological disease. An estimated 15% of the overall population was affected with migraine [1, 2]. The most common symptoms of migraine include photophobia, phono-phobia, nausea, vomiting, tinnitus, dizziness,

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and cognitive impairment [3]. Modern medicines for migraine treatment mainly belong to non-steroidal anti-inflammatory drugs (NSAIDs), triptans (5-HT receptor agonists), calcitonin gene-related peptide (CGRP) receptor antagonists, dopamine receptor antagonists, as well as beta-blockers, anti-depressants, anti-convulsants, monoclonal antibodies against CGRP molecules, and receptors. However, several of these medicines have unsatisfactory treatment efficacy and unpleasant adverse effects, as well. Hence, a demand for the development of alternative migraine therapies to improve the treatment outcome of these anti-migraine medicines is urgent.

The Duliang herbal formula, a traditional medicine for the anti-migraine effect, was fully recorded and described in folk Chinese medicine literature as well as in Chinese Pharmacopoeia. The DHF includes two components, *Angelica dahurica* radix (ADR) and *Ligusticum chuanxiong* rhizome (CXR), with a ratio of 4:1 w/w [4].

The traditional Chinese medicine (TCM) formula containing a number of active substances was generally prepared with one or several herbal components. Based on the fundamental theory that multiple ingredient medicinal formula aims at multiple rather than single targets, the network pharmacology approach was developed using the updated bioinformatic models, tools, and data [5]. The network pharmacology approach constructed and topologically analyzed a net with nodes as herbs, compounds, targets, and diseases and edges as the relationship between nodes. The topologically analyzing results contribute to clearer insights into the multiple therapeutic effects of multiple ingredients and multidrug targets and their functions [6]. Additionally, chromatographic analysis of herbal formulas significantly supported in determining the chemical composition of herbs, which was considered as the basis for quality control of herbs [7].

In order to extend our findings published about the network pharmacology approach for an explanation of the anti-migraine mechanism of DHF, this study aims to: *Develop an approach of chromatogram similarity analysis to explore the potential biomarkers of DHF for migraine treatment [8].*

MATERIALS AND METHODS

1. Materials

Samples of ADR and CXR were collected from different companies in different areas of Korea in August 2021.

2. Methods

** Sample preparation:*

For DHF preparation, first, dried ADR (1g) was cut into small pieces using hand scissors and combined with 10mL of fermented ethanol (85%, v/v) and ultrasonicated (45kHz, 30min) to extract compounds interested. The extraction process of ADR was repeated 3 times. The resultant extract was collected. The similar extraction of CXR was implemented using fermented ethanol (90%, v/v). The DHF extract is a mixture of two herbal extracts with a ratio of 4:1 (v/v) of ADR and CXR extracts, respectively.

** Chromatographic conditions:*

HPLC analyses were performed on a Shimadzu 20AD HPLC system (Shimadzu, Japan) consisting of a quaternary solvent delivery system, an on-line degasser, an autosampler, a column temperature controller, and a diode array detector interfaced with a Shimadzu 8040 triple quadrupole mass spectrometer (Shimadzu, Japan). The analytical column was a Hector-C1 column (4.6 x 250mm, 5µm), and the column temperature was maintained at 35^0C. The mobile phase A: 0.1% formic acid (v/v) in water and B: acetonitrile were used. A gradient program was used as follows: 0 - 10min, 10% B; 10 - 120min, 10 - 95% B; 120 - 125min, 95% B, with a hold time of 10min, which was together with the flow rate: 0.5 $mL.min^{-1}$, injection volume: 10µL.

** Diode array detection (DAD):*

The signal of absorbance wavelength was detected at 270nm. Setting the acquisition range from 190 - 400nm, the diode array detection allowed the acquisition of absorbance spectrum for each compound, which induces the identification of compounds via comparison with relevant standards or the tentative prediction of substances via literature information of maximum absorption spectrum.

** Electrospray ionization - tandem mass spectroscopy detection (ESI-MS/MS):*

The HPLC conditions described above were used for HPLC/QqQ MS analysis. LC-ESI-MS system with scan mode in both positive and negative

modes was utilized for peak identification. To verify the production of each peak in the samples, a survey on product ions was performed. The other parameters were set: An interface voltage of + 4.5kV for the positive mode and -3.5kV for the negative mode; a desolvation line temperature: 250ºC; flow rate of drying gas: 15 L/min; nebulizing gas: 3 L/min; desolvation line temperature: 250ºC; heat block temperature: 400ºC.

** Similarity evaluation of chromatographic data of DHF batches:*

In order to establish a representative chromatogram, all batches were analyzed by the present methods, and the obtained data were exported in AIA format (in file *.cdf) via chromatogram processing software LabSolutions (Shimadzu). Subsequently, the tool Computer-Aided Similarity Evaluation System for Chromatographic Fingerprints of Chinese Medicines was used to process these output chromatogram data.

RESULTS AND DISCUSSION

1. Exploration of potential anti-migraine compounds in DHF via network pharmacology approach [8]

Figure 1 shows the analysis result of the active compound-target-pathway network with the enriched KEGG pathways, targets, and compounds that regulated these pathways. The degree of a node in this network was reflected via the size of the node. The larger size of the node in the network, the more significant it was, which exhibits the significance of a compound, target, or pathway in the therapeutic or pathogenetic mechanism of DHF against migraine. The top 20 KEGG pathways (significance degree $p < 0.01$) and their corresponding targets and compounds were constructed in network visualization, which was displayed in figure 1. Based on topology analysis of the network, MAPK1 showed the largest size; therefore, MAPK1 was considered as a core target. In addition, other targets showed their size relatively large, such as PIK3CA, EGFR, RELA, NOS2, and DRD2. These gene targets were used to retrieve the association with active compounds. Additionally, the consideration of a compound as a potential quality marker in DHF against migraine was based on the degree of compound in the network, shown in table 1.

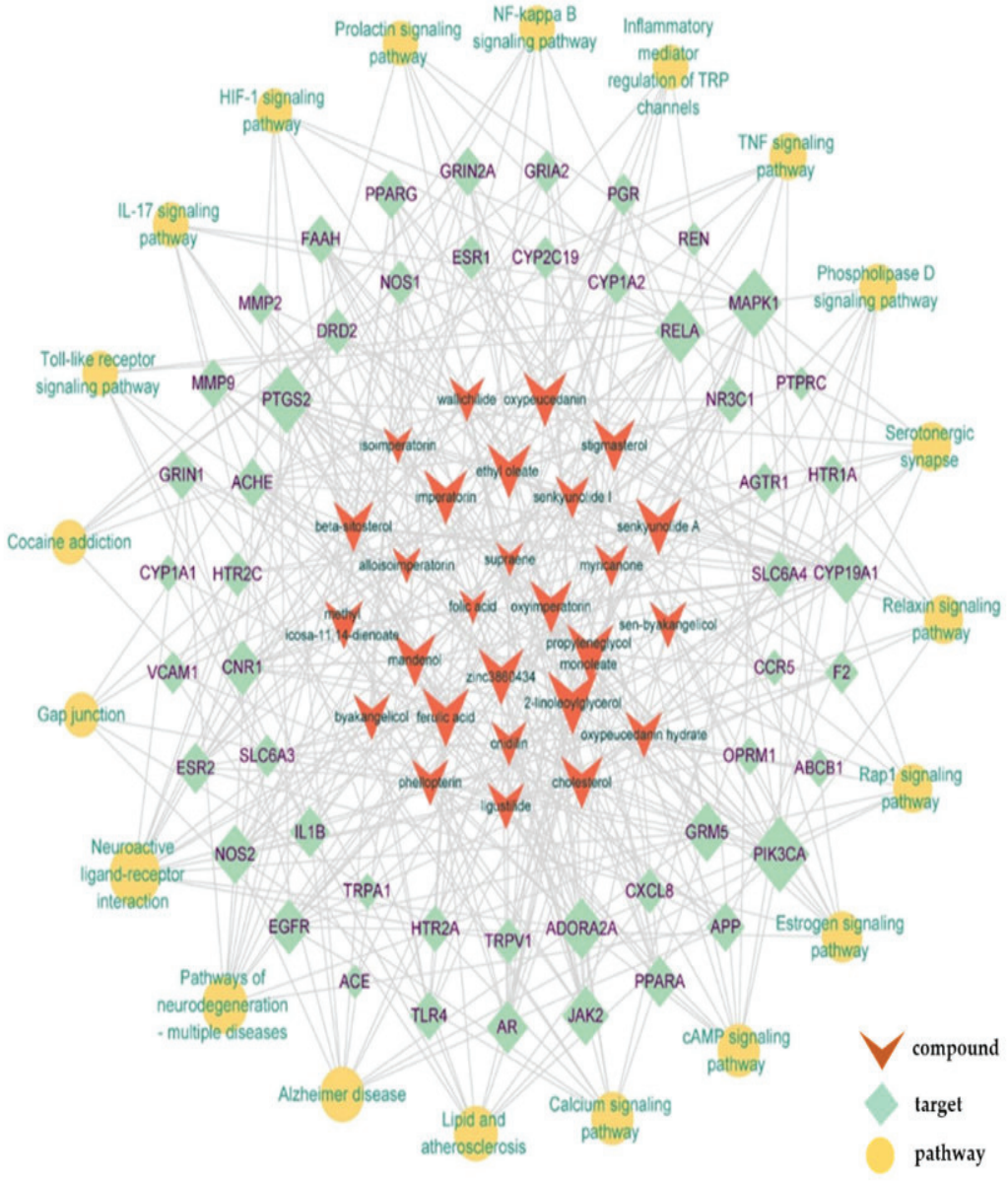

Figure 1. The active component-target-pathway network.

These active ingredients showed a high correlation with the 10 core targets. RELA, MAPK1, and AKT1 were involved in most of the pathways, suggesting that DHF may exert its effects through these targets.

Table 1. Degree of compound nodes in the Compound-Target-Pathway network of the DHF for anti-migraine effect.

Herb	Compound	Degree
ADR	2-linoleoylglycerol	15
ADR	propyleneglycol monoleate	13
CXR	ferulic acid	13
ADR	zinc3860434	12
CXR	senkyunolide A	12
ADR	ethyl oleate	11
ADR	imperatorin	11
CXR	oxypeucedanin	11
ADR	cholesterol	10
ADR	oxyimperatorin	10
ADR	stigmasterol	10
ADR/ CXR	mandenol	10
ADR	beta-sitosterol	9
ADR	methyl icosa-11,14-dienoate	8
ADR	byakangelicol	7
ADR	phellopterin	7
ADR	oxypeucedanin hydrate	7
ADR	sen-byakangelicol	7
CXR	ligustilide	7
ADR	cnidilin	6
CXR	wallichilide	6
CXR	myricanone	5
CXR	senkyunolide I	5
ADR	alloisoimperatorin	2
ADR	isoimperatorin	2
ADR	supraene	1
CXR	folic acid	1

2. Chemical profiling of DHF using DAD and MS/MS spectrometry

According to the experimental assay mentioned in part 2. Method via HPLC-DAD-MS/MS method, the main components in DHF, including the extract mixture of ADR and CXR with a ratio of 4:1 (w/w), were structured on the basis of the order of compound retention time, accurate mass, fragment information, mass spectrometry fragmentation laws, and other information [9, 10]. A total of 21 compounds were identified or tentatively characterized *(Table 2).*

Table 2. Chemical identification of components in DHF via HPLC-DAD-MS/MS.

Peak no.	t_R (min)	λ_{max} (nm)	Precursor ion (m/z)	Product ions (m/z)	Identification
1	28.628	218; 271	355.00 $[M+H]^+$	163.05; 117.15; 135.00; 89.05; 145.05	Chlorogenic acid *
2	34.970	218; 237; 326	353.20 $[M-H]^-$	172.90; 178.90; 134.85; 190.90; 93.00	Cryptochlorogenic acid *
3	38.401	217; 239; 323	181.00 $[M+H]^+$	89.05; 117.10; 141.30; 77.10	Caffeic acid *
4	48.009	218; 233; 323	194.90 $[M+H]^+$	176.95; 89.10; 145.00; 116.95; 194.90	Ferulic acid *
5	53.882	276	224.95 $[M+H]^+$	207.15; 91.05; 164.95	Senkyunolide I *
6	56.563	218; 276; 314	247.05 $[M+Na]^+$	175.00; 147.00; 119.05; 131.00; 159.05	Senkyunolide H
7	60.799	222; 249; 265; 310	304.90 $[M+H]^+$	202.90; 147.00; 91.05; 131.00; 59.10	Oxypeucedanin hydrate *
8	61.882	223; 268; 313	317.00 $[M+H]^+$	217.95; 175.00; 187.95; 202.95; 230.95	Apaensin
9	69.047	245; 292; 324	186.95 $[M+H]^+$	131.05; 115.10; 77.15; 142.95; 51.20	Psoralen *

Peak no.	t_R (min)	λ_{max} (nm)	Precursor ion (m/z)	Product ions (m/z)	Identification
10	71.001	218; 246; 302	217.05 $[M+H]^+$	174.00; 201.95; 118.00; 145.05; 160.95	Xanthotoxin
11	75.985	223; 248; 267; 311	216.90 $[M+H]^+$	201.90; 174.00; 89.05; 118.05; 90.00	Bergapten *
12	77.694	218; 247; 299	287.00 $[M+H]^+$	203.05; 172.90; 156.95; 147.00; 129.00	Pabulenol
13	81.689	223; 268; 311	334.05 $[M+NH_4]^+$	230.90; 299.05; 316.95; 233.00; 174.95	Byakangelicol *
14	82.505	220; 249; 307	287.00 $[M+H]^+$	203.00; 59.05; 85.00; 146.95; 57.10	Oxypeucedanin *
15	86.189	278	192.95 $[M+H]^+$	91.05; 137.05; 147.10; 77.10; 93.05	Senkyunolide A *
16	92.152	218; 248; 300	288.00 $[M+NH_4]^+$	202.95; 271.05; 146.95; 69.15; 91.10	Imperatorin *
17	94.099	205; 281; 325	190.95 $[M+H]^+$	172.95; 91.00; 77.05; 115.00; 117.10	Ligustilide *
18	95.254	224; 268; 312	301.00 $[M+H]^+$	217.90; 189.80; 230.10; 162.00; 173.00	Phellopterin
19	97.318	224; 245; 267; 309	301.05 $[M+H]^+$	172.95; 218.00; 233.00; 162.95; 189.85	Cnidilin
20	98.418	222; 250; 308	270.90 $[M+H]^+$	202.90; 146.95; 91.10; 131.05; 41.15	Isoimperatorin *
21	99.079	223; 268; 311	245.05 $[M+H]^+$	186.95; 131.00; 115.15; 245.15; 214.95	Suberosin

** Confirmed with identification of reference substances:*

Figure 2 shows the representative chromatogram of DHF. Comparing the mass spectra and retention times of the components of DHF with those of the corresponding standards, peaks 1, 2, 4, 5, 7, 9, 11, 13, 14, 15, 16, 17, and 20 could be unambiguously identified as chlorogenic acid (1), cryptochlorogenic acid (2), ferulic acid (4), senkyunolide I (5), oxypeucedanin hydrate (7), psoralen (9), bergapten (11), byakangelicol (13), oxypeucedanin (14), senkyunolide A (15), imperatorin (16), ligustilide (17) and isoimperatorin (20), respectively.

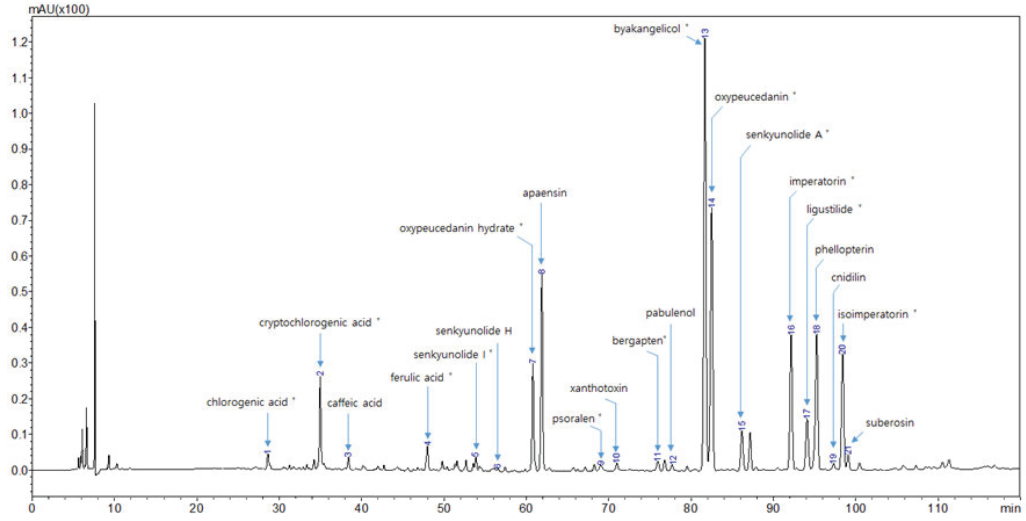

Figure 2. The representative chromatogram of chemical profile in DHF at 270nm *(*compounds confirmed with corresponding standards).*

3. Similarity analysis of chromatographic data of DHF batches and exploration of potential biomarkers

The similarity analysis of chromatographic data of the DHF (HPLC-UV detection at 270nm) was carried out via the tool "Similarity Evaluation System of chromatographic fingerprint of TCM" (released version 2004A). This software was issued by the Chinese pharmacopeia committee.

The HPLC fingerprints of the 26 samples are shown in figure 3. There were 22 common peaks, and the sum of peak areas of all shared peaks was > 90%. To compare with the standards, nine peaks were identified, namely ferulic acid, senkyunolide I, oxypeucedanin hydrate, byakangelicol, oxypeu-cedanin, senkyunolide A, imperatorin, ligustilide, isoimperatorin.

The chromatographic peaks were automatically matched, and the reference fingerprint chromatography of 25 batches was produced by the median method. The similarities between the chromatograms of these samples (A1L1 - A5L5) were 0.923; 0.990; 0.961; 0.959; 0.966; 0.948; 0.992; 0.951; 0.971; 0.924; 0.938; 0.954; 0.974; 0.935; 0.934; 0.958; 0.968; 0.921; 0.992; 0.944; 0.977; 0.936; 0.972; 0.965; 0.981; and 0.958, respectively *(Figure 3).*

Based on the network pharmacology, similarity analysis results, and literature search, we finally suggested nine active compounds, namely ferulic acid, senkyunolide I, oxypeucedanin hydrate, byakangelicol, oxypeucedanin, senkyunolide A, imperatorin, ligustilide, isoimperatorin as potential biomarkers for quality control of DHF.

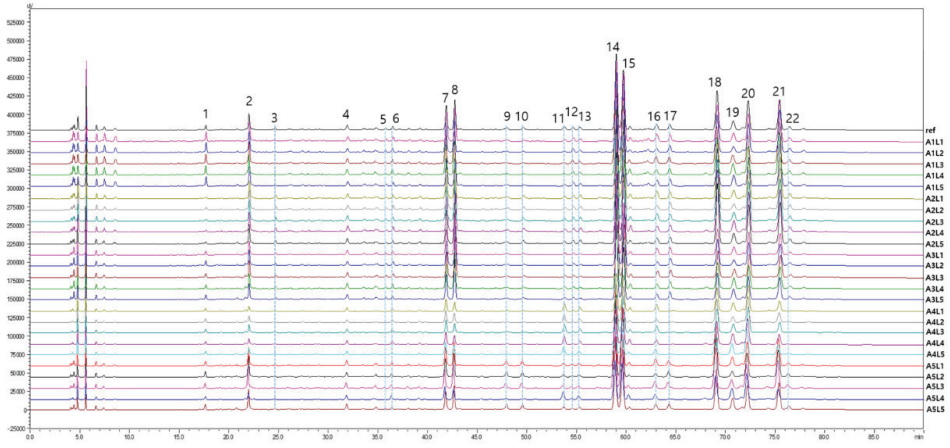

Figure 3. The chromatograms of 25 batches of Duliang formula were monitored at 270nm.

CONCLUSION

Based on the network pharmacology, this study revealed that the DHF for anti-migraine activity functions via regulating multiple targets and pathways in the pathological genesis and progression of migraine. In the pathophysiological pathways of migraine, the most active components of DHF, such as imperatorin, senkyunolide I, senkyunolide A, phellopterin, byakangelicol, cnidilin, ferulic acid, senkyunone, and wallichilide, byakangelicol, ligustilide, isoimperatorin, oxypeucedanin, and oxypeucedanin hydrate, was expressed in various associations. Additionally, in combination with experiments of

chemical analysis using a LC-DAD-MS/MS approach, the chemical profile of DHF was identified, which proposes nine quality markers of DHF. In summary, this study will bring a clearer basis for more in-depth research, for an increase in the quality, and a more widespread clinical application of DHF for migraine.

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